Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5841–5844, June 1995
Evolution

Exponential increases of RNA virus fitness during large population transmissions

ISABEL S. NOVELLA*, ELIZABETH A. DUARTE*†, SANTIAGO F. ELENA‡, ANDRES MOYA‡, ESTEBAN DOMINGO§, AND JOHN J. HOLLAND*

*Department of Biology and Institute for Molecular Genetics, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116; ‡Departament de Genetica i Servei de Bioiformatica, Facultat de Biologia, Universitat de Valencia, E-46100 Burjassot, Valencia, Spain; and §Centro de Biologia Molecular "Severo Ochoa" (Consejo Superior de Investigaciones Cientificas), Universidad Autonoma de Madrid, Cantoblanco, E-28049 Madrid, Spain

Communicated by Manfred Eigen, Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Germany, March 27, 1995

ABSTRACT **The great adaptability shown by RNA viruses is a consequence of their high mutation rates. Here we investigate the kinetics of virus fitness gains during repeated transfers of large virus populations in cell culture. Results always show that fitness increases exponentially. Low fitness clones exhibit regular increases observed as biphasic periods of exponential evolutionary improvement, while neutral clones show monophasic kinetics. These results are significant for RNA virus epidemiology, optimal handling of attenuated live virus vaccines, and routine laboratory procedures.**

RNA viruses are highly mutable and form complex quasispecies populations as defined by Eigen and colleagues (1–4). Quasispecies or "mutant swarms" of RNA viruses evolve thousands- to millionsfold faster than DNA-based organisms (5–9). This provides an invaluable tool to perform evolutionary studies that, for the latter, would take eons. Evolution of RNA viruses depends upon environmental selective forces and random drift (6, 10, 11). Examples are human immunodeficiency virus 1 (11), hepatitis C virus (12), and foot-and-mouth disease virus (13), all of which can replicate and evolve rapidly and continuously in infected individuals. Because the behavior of quasispecies populations is important for an understanding of RNA virus disease and epidemiology, quantitative studies of virus populations and population genetics are needed. We have developed a relative fitness assay to enable quantitative analysis of RNA virus population behavior (14). This employs genetically marked mutants that are mixed with wild-type virus (as an internal standard), and these mixed RNA virus quasispecies are allowed to compete during replication in a series of repeated transfers in cell culture. The changing ratios of genetically marked virus to wild-type virus allow determination of relative fitness vectors and relative fitness values (W) per passage. For the wild-type virus employed as the internal control, fitness is assigned a neutral value ($W = 1.0$) because it is the parental standard virus clone from which all of the genetically marked clones have been derived. The marked clones are monoclonal antibody-resistant mutants (MARMs), and their fitness is measured after replicative competition passages in a constant cell culture environment (14).

It was observed (15–18) that whenever selection does not have the opportunity to act, as during repeated genetic bottleneck transfers of a MARM of vesicular stomatitis virus (VSV) or a marked mutant of an RNA bacteriophage, the high mutation rates lead to loss of virus fitness. Genetic bottleneck passages involve repeated transfers of only one or a few virions, and loss of fitness results from gradual stochastic accumulation of deleterious mutations in accord with Muller's ratchet theory (19, 20). Muller (19) had predicted that when an asexual population is small and the mutation rate is high, the population will decline in fitness due to the accumulation of deleterious mutations in a "kind of irreversible ratchet mechanism." In contrast, it has been observed that repeated transmissions of large RNA virus populations from host to host in a constant environment leads to significant increases in mean population fitness. This occurs because replicative competition allows selection to operate, causing loss of inferior genomes and accumulation of more-fit genomes (21). More-fit mutants predictably gain rapid ascendancy in virus populations (10), and the "cloud of mutants" in quasispecies populations will be "guided" through "sequence space" by natural selection as elaborated by Eigen and colleagues (1–4). Sequence space is a ν-dimensional abstract world (where ν is the genome length in bases for an RNA virus). Sequence space has $>4^{11,000}$ dimensions for an RNA genome exceeding 11 kb, and most of this unimaginably immense space is devoid of life. The evolutionary challenge for living entities is to find, and move within, those relatively diminutive regions of sequence space that are viable and adaptative. Despite the incomprehensible vastness of sequence space, there is high connectivity from one genomic sequence to others (a finite Hamming distance), and this allows quasispecies mutant swarms to "climb uphill" (be positively selected to move increasingly higher in those regions of sequence space with greater adaptative value) (1–4).

Hill climbing in sequence space, as with adaptative peak ascensions in the earlier adaptative landscape paradigm of Wright (22, 23), represents the fundamental action of Darwinian natural selection. In the case of RNA virus quasispecies evolution, adaptative movements in sequence space have important biological, medical, and epidemiological consequences. In the present study, we have examined carefully the kinetics of fitness acquisition during repeated transmission of large virus populations under constant environmental conditions, and we observed remarkable exponential gains in fitness. The viral populations tested were VSV MARM clones with either low starting fitness or approximately neutral fitness. Because we always started with clones (progeny of single virus particles initiating a virus plaque), all of the exponential fitness gains shown below must inevitably represent adaptative uphill climbs of RNA virus quasispecies mutant clouds through sequence space (3).

MATERIALS AND METHODS

BHK_{21} cells and HeLa cells were grown as cell monolayers in Eagle's minimum essential medium containing heat-inactivated (60°C, 30 min) bovine calf serum. The virus employed was the Mudd–Summers strain (Indiana serotype) of VSV (24). Our wild-type virus is a population derived from a clone, and all the genetically marked MARMs are subclones from

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MARM, monoclonal antibody-resistant mutant; VSV, vesicular stomatitis virus.

†Present address: Institute of Biochemistry, University of Lausanne, CH-1066, Epalinges, Switzerland.

this wild-type clone. Some clones (D, N, and C) were previously subjected to genetic bottleneck (plaque-to-plaque) transfers that reduced their relative fitness (17, 21, 25). They are clonal pools prepared from a plaque picked after the final bottleneck passage. MARM U is a neutral (surrogate wild type) subclone of the wild-type clone recovered from an isolated plaque grown in the presence of I1 monoclonal antibody (14). Hybridoma cells were kindly provided by L. Lefrancois (26, 27) and concentrated I1 monoclonal antibody was prepared in cell cultures exactly as described (28). Transfers of large virus populations and competition assays were done as described (14, 17, 18, 21, 28–30). Briefly, mixtures of MARM populations and wild-type virus were seeded on BHK_{21} monolayers and allowed to compete during consecutive transfers at 37°C (until the cytopathic effect was complete). The virus initial mixture and yields after each transmission cycle were subjected to triplicate plaque assays in BHK_{21} cells (with and without monoclonal antibody) to determine MARM/wild-type ratios. In the present study, initial MARM/wild-type ratios varied from 0.8:1 to 40:1. Fitness values were obtained as described (31). The VSV Mudd–Summers wild type is assigned to relative fitness of 1.0 (as the internal fitness standard) (14).

Each experiment started with one of the genetically marked MARM clones of VSV, termed MARM clones D, C, U, and N. To analyze the evolution of virus fitness, we repeatedly transferred aliquots containing large populations of each clone (10^5 to 10^6 infectious particles) up to 100 times in cell culture (by using 2×10^6 to 3×10^6 cells per infection; i.e., a multiplicity of infection <1.0), and we determined fitness of intermediate passages in each case as described (14, 17, 18, 21, 28, 29). When necessary, virus clones and transferred populations were stored frozen at −70°C or at −85°C until employed for experiments or fitness assays.

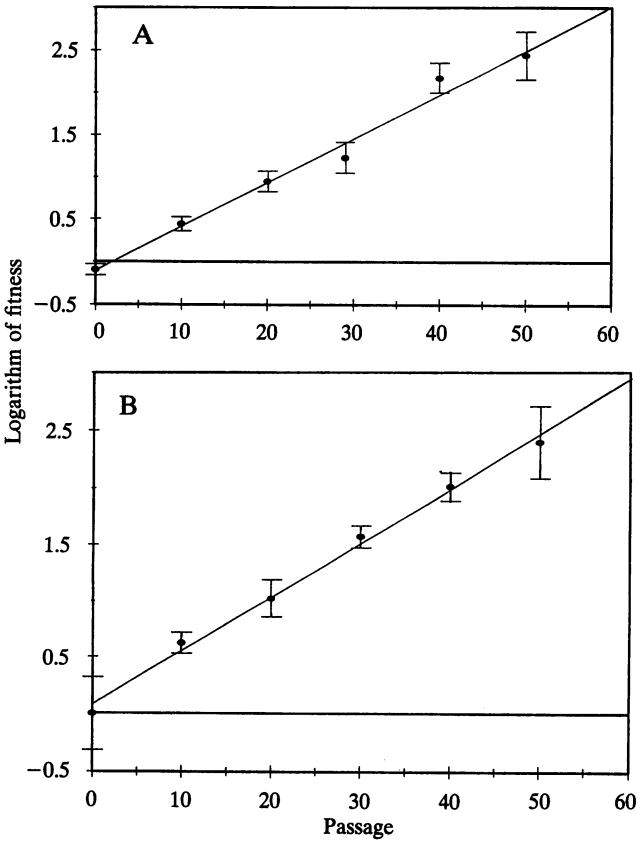

FIG. 1. Kinetics of fitness changes during transmission of neutral MARM clones C (*A*) and U (*B*). Fitness competitions were performed as described (14, 28).

RESULTS

When we carried out repeated transmission of large populations (10^5 to 10^6 plaque-forming units) of neutral fitness virus clones C (initial $W = 0.93 \pm 0.02$) and U (initial $W = 1.0 \pm 0.2$) in BHK_{21} cells, we observed rather regular exponential increases in mean fitness of these quasispecies populations (Fig. 1). Repeated transfers of large virus populations of clone D (initial $W = 0.30 \pm 0.04$) in HeLa cells (Fig. 2) gave similar results. However, for this virus clone that had initially low fitness, the resulting exponential increases exhibited two distinct phases of continuous evolution. During the first phase, fitness gains occurred rapidly until reaching approximate neutrality. Beyond this point, the exponential fitness increases continued, but at a lower rate. Fig. 2*A* shows the individual fitness vectors at a number of passages beginning with passage zero (the initial mixture of clone D and wild-type control virus) and passage 3 and ending with passage 80. Fig. 2*B* plots the same transfer series cumulatively. Adaptation was rather spe-

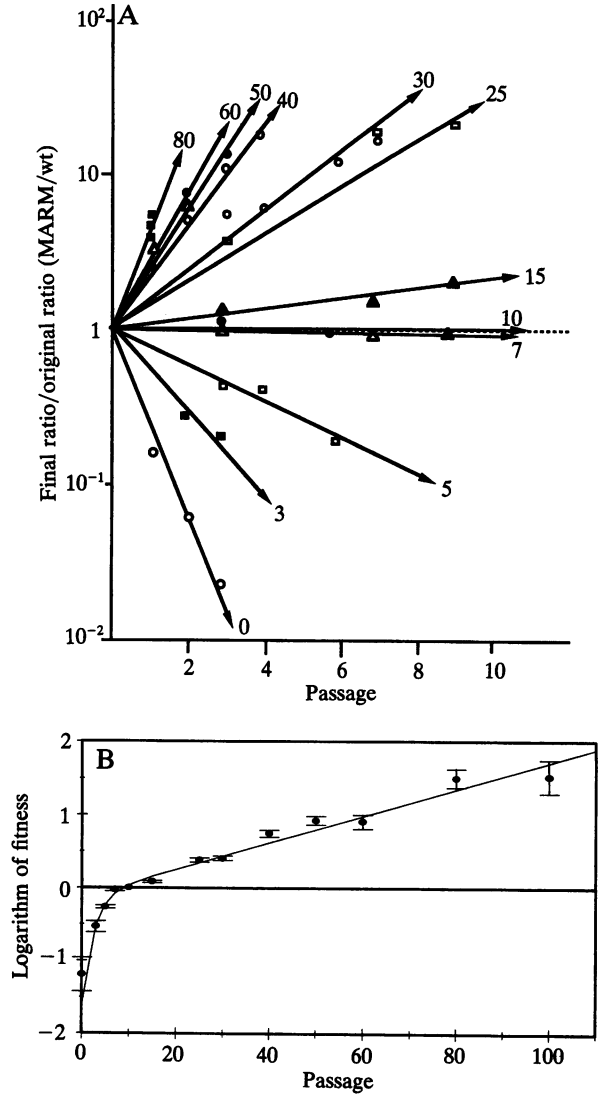

FIG. 2. Relative fitness changes during transmission of MARM clone D on HeLa cells. Experiments were performed as described in Fig. 1, except that transmissions were carried out in HeLa cells. (*A*) Individual fitness vectors at each indicated competition passage number during 80 consecutive passages (the passage number tested is indicated next to the arrowhead of the corresponding vector). wt means wild type. (*B*) Kinetics of fitness changes during the entire transmission series. Each point corresponds to the relative fitness value (*W*) derived from each individual vector shown in *A*.

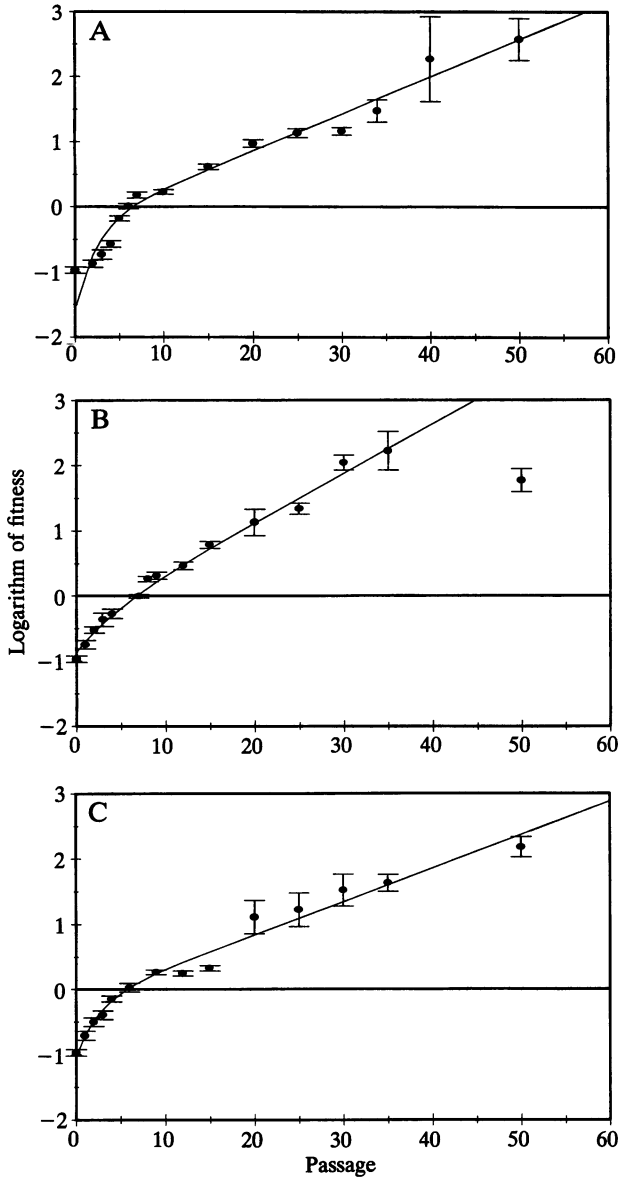

FIG. 3. Relative fitness changes during transmission of MARM clone N. *A–C* represent the results of each one of the three replicas carried out.

cific, because the 100th passage of MARM D in HeLa cells showed significantly lower fitness values during competition on canine MDCK and mouse L-929 monolayers (data not shown). Next we examined another low-fitness clone, clone N, and obtained very similar results (Fig. 3). Three replicates of clone N transfers were carried out, and the resulting exponential slopes and absolute values for fitness increases were equivalent but differed slightly among each of the three replicate transfer series. This was to be expected, because such fitness increases are driven by stochastic processes of mutation, random sampling, and selection (10). However, stochastic generation of any individual variant (or variant subsets) with higher fitness is partially buffered by the complex population dynamics of a quasispecies mutant cloud moving uphill to more adaptative regions (not fixed points) in sequence space (3, 32). This probably acts to dampen tendencies toward sudden discontinuous changes in mean quasispecies population fitness. At the same time, lower fitness genomes are gradually eliminated by natural selection, and their mean rate of extinction masks the varying rate of loss of individual classes of variants. The clear result of all selective processes during these passages is exponential increases of mean quasispecies fitness during repeated transmission in a constant environment.

DISCUSSION

Although RNA virus quasispecies have long been known to be highly adaptable (3–8, 10), the results reported here show an adaptative evolutionary capacity that overwhelms speculation. It should be stressed that each series came from a single infectious particle that rapidly generated all mutations needed for the remarkable gains in fitness observed (nearly 5000% after 50 passages of clone N, series A). Values reported for *Escherichia coli* fitness gains during adaptation to new culture conditions are 8% in 400 generations (33) and around 37% in 2000 generations (34, 35). Another remarkable difference between *E. coli* and VSV evolution is that *E. coli* fitness changes can be explained by an hyperbolic model (34), whereas VSV evolution follows exponential kinetics in the form $\log w_t = A + Bt - Ce^{-Dt}$, where w_t is the mean fitness at passage t and A, B, C, and D are empirical parameters for the model fit obtained by the generalized minimum squares method (Table 1). Nevertheless, both models share important characteristics, particularly the continuous regular fitness gains and the biphasic shape of the curves. Note however in Table 1 that our RNA virus data show very poor fit (right hand column) to the hyperbolic model for *E. coli* (33–35). Any hyperbolic model to fit our data would have to be based upon a logarithmic transformation of experimental fitness values. This difference in adaptative rates and modes might be due to differences in mutation rates and genomic sizes in DNA-based and RNA-based genomes (6, 8, 38).

This exponential mode of fitness variation is in agreement with a model previously proposed by Gabriel *et al.* (36) and Lynch *et al.* (37). In their model, the kinetics of mutation accumulation (i.e, mean fitness) is described in terms of population genetic parameters such as mutation rate (μ), selection coefficients (s) for new mutants, and carrying capacity (K) of the system (Table 1). Their model was designed to

Table 1. Estimated values for parameters corresponding to the exponential model

MARM clone	*A*	*B*	*C*	*D*	$R^2(E)$	$R^2(H)$
C	−0.1049 ± 0.1362	0.0522 ± 0.0033	—	—	0.9846	0.235
U	0.0786 ± 0.0709	0.0476 ± 0.0017	—	—	0.9950	0.288
D	−0.1128 ± 0.1198	0.0182 ± 0.0013	1.5195 ± 0.5089	0.3893 ± 0.0440	0.9726	0.162
N(A)	−0.2827 ± 0.1688	0.0571 ± 0.0042	1.2858 ± 0.8754	0.4045 ± 0.1409	0.9596	0.249
N(B)	−0.3822 ± 0.1014	0.0754 ± 0.0038	0.4813 ± 0.1058	0.1963 ± 0.0396	0.8886	0.258
N(C)	−0.1903 ± 0.2068	0.0511 ± 0.0057	0.8411 ± 0.1371	0.3719 ± 0.0338	0.9783	0.244

Estimates were obtained by means of numeric computations using the Levenberg–Marquard method. $R^2(E)$ shows the regression values for the exponential model. $R^2(H)$ shows the regression values for the hyperbolic model described by Lenski and coworkers (33–35) for *E. coli* fitness gains. The correspondence between the estimated parameters and the theoretical ones (36, 37) is as follows: $A = \mu s^2\alpha^2/(1 - \alpha)$, $B = s\mu(1 - s)/(1 + Ks)$, $C = A - \ln w_o$, and $D \approx -\ln \alpha$, where $\alpha = (1 + 1/K)(1 - s)$ and w_o is the initial fitness value. This model of exponential fitness gains fit [$R^2(E)$] the exponential model of fitness variation proposed by Gabriel *et al.* (36) and Lynch *et al.* (37) very well but fit the hyperbolic model [$R^2(H)$] very poorly.

explain accumulation of deleterious mutations with consequent loss of fitness. However, in the present work we are observing the opposite phenomenon: gains in fitness, apparently as a consequence of the accumulation of advantageous mutations. It should be noted that fitness gains of low-fitness MARM populations (clones D and N) exhibited very similar kinetics in the two cell lines (BHK_{21} or HeLa) used in the experiments. Likewise, virus clones passaged in the same cell line (BHK_{21}) but varying in initial fitness (neutral MARMs C and U or low-fitness MARM N) differed in having either single-phase or two-phase kinetics of fitness increase. Regardless of the cell type employed, the form of the fitness increases can be interpreted as follows: initially, when the fitness of the original clone is low (as for N and D, mutants), all possible advantageous mutations improve fitness and become fixed in the population very rapidly. When the mean fitness of the population reaches a value near neutrality, only those subsets of possible mutations that have a very high selective coefficient would modify the mean fitness value and become fixed rapidly in the population (at a rate close to $\mu s/K$). This change in the subsets of possible beneficial mutations could explain both the break in the speed of optimization for the low-fitness MARM clones and the constant exponential fitness increases observed for neutral MARM clones. Although the neutral value of wild type was assigned to be 1, the kinetics we observed in this study suggest that "neutrality" may be biologically meaningful. This is supported by our observation (data not shown) that two other independent wild-type isolates from bovine sources have a fitness very close to that of the wild-type strain used here (24).

Obviously, there must be inevitable limits to fitness gains during repeated transmission, but there is no precise value at which fitness becomes maximal, and we regularly observe much higher scatter of replicate fitness values at extremely high fitness levels. As virus populations evolve even in a constant environment, they may shift at irregular intervals to neighboring peaks in the paradigm of Wrightian adaptative landscapes (22, 23) or, more accurately, to new areas of sequence space as elaborated by Eigen and colleagues (1–4). Our findings have some practical implications. (*i*) Repeated transmission of large virus inocula from one individual to another during a virus outbreak or epidemic is more likely to select highly fit viruses with greater capacity to replicate rapidly and outrace immune responses. This is in addition to the inherently greater risk posed by larger infectious doses. (*ii*) When attenuated live virus vaccine clonal seed stocks are prepared, each additional passage beyond the seed stock poses increasing (exponential) potential for selection of more robust (and sometimes more virulent) variants [see review by Wimmer *et al.* (7) of the complexity of poliovirus virulence and vaccine attenuation or virulence]. (*iii*) Routine handling of all RNA virus stocks for research and other purposes should take account of these regular exponential changes in biological properties during passage. (*iv*) In the context of any "newly emerged" RNA virus disease of humans, initial periods and average doses of person-to-person transmission would be most critical, as virus populations adapt to infect a new host.

Finally, it should be emphasized that all of the above studies were carried out in a constant environment by using single host cell types. Adaptative fitness increases on BHK_{21} cells under these conditions were observed previously to be deadaptive for virus replication in a neural environment after intracerebral inoculation of mice (14). Similarly, the MARM D clone after 100 passages in HeLa cells showed significantly lower fitness on a canine or mouse cell line. This points out that increasingly adaptive uphill climbs in sequence space may often be highly specific for the selecting environment. What is uphill in one environment can be downhill in another, although not necessarily.

We thank E. Bussey for excellent technical assistance and Dr. Leo Lefrancoise for I1-producing hybridomas. Work in La Jolla was supported by the National Institute of Allergy and Infectious Diseases (Grant AI-14627). Work in Madrid and Valencia was supported by Grant DGICYT PB91-00510. Work in Madrid was also supported by Fundacion Ramon Areces. I.S.N. and S.F.E. acknowledge fellowships from Comisio Interdepartamental de Recerca i Innovacio Tecnologica (Generalitat de Catalunya) and Conselleria d'Educacio i Ciencia (Generalitat Valenciana), respectively.

1. Eigen, M. & Schuster, P. (1979) *The Hypercycle: A Principle of Natural Self-Organization* (Springer, Berlin).
2. Eigen, M. & Biebricher, C. K. (1988) in *RNA Genetics*, eds. Domingo, E., Holland, J. J. & Ahlquist, P. (CRC, Boca Raton, FL), pp. 211–245.
3. Eigen, M. (1992) *Steps Towards Life: A Perspective on Evolution* (Oxford Univ. Press, New York).
4. Eigen, M. (1993) *Sci. Am.* **269,** 42–49.
5. Holland, J. J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S. & VandePol, S. (1982) *Science* **215,** 1577–1585.
6. Domingo, E. & Holland, J. J. (1994) in *The Evolutionary Biology of Viruses*, ed. Morse, S. (Raven, New York), pp. 161–183.
7. Wimmer, E., Hellen, C. U. T. & Cao, X. (1993) *Annu. Rev. Genet.* **27,** 353–436.
8. Drake, J. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 4171–4175.
9. Strauss, J. H. & Strauss, E. G. (1988) *Annu. Rev. Microbiol.* **42,** 657–683.
10. Coffin, J. H. (1992) *Curr. Top. Microbiol. Immunol.* **176,** 143–164.
11. Wain-Hobson, S. (1994) in *The Evolutionary Biology of Viruses*, ed. Morse, S. S. (Raven, New York), pp. 185–209.
12. Martell, M., Esteban, J. I., Quer, J., Genesca, J., Weiner, A., Esteban, R., Guardia, J. & Gomez, J. (1992) *J. Virol.* **66,** 3225–3229.
13. Domingo, E., Escarmis, C., Martinez, M. A., Martinez-Salas, E. & Mateu, M. G. (1992) *Curr. Top. Microbiol. Immunol.* **176,** 33–47.
14. Holland, J. J., de la Torre, J. C., Clarke, D. K. & Duarte, E. (1991) *J. Virol.* **65,** 2960–2967.
15. Chao, L. (1990) *Nature (London)* **348,** 454–455.
16. Chao, L. (1994) in *The Evolutionary Biology of Viruses*, ed. Morse, S. S. (Raven, New York), pp. 233–250.
17. Duarte, E., Clarke, D., Moya, A., Domingo, E. & Holland, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6015–6019.
18. Duarte, E. A., Clarke, D., Elena, S. F., Moya, A., Domingo, E. & Holland, J. (1993) *J. Virol.* **67,** 3620–3623.
19. Muller, H. J. (1964) *Mutat. Res.* **1,** 2–9.
20. Haigh, J. (1978) *Theor. Popul. Biol.* **14,** 251–267.
21. Clarke, D., Duarte, E., Moya, A., Elena, S. F., Domingo, E. & Holland, J. (1993) *J. Virol.* **67,** 222–228.
22. Wright, S. (1931) *Genetics* **16,** 97–159.
23. Wright, S. (1982) *Evolution* **36,** 427–443.
24. Mudd, J. A. & Summers, D. F. (1970) *Virology* **42,** 328–340.
25. Novella, I. S., Elena, S. F., Moya, A., Domingo, E. & Holland, J. J. (1995) *J. Virol.* **69,** 2869–2872.
26. Lefrancois, L. & Lyles, D. (1982) *Virology* **121,** 168–174.
27. Lefrancois, L. & Lyles, D. (1982) *Virology* **121,** 157–167.
28. Duarte, E. A., Novella, I. S., Ledesma, S., Clarke, D. K., Moya, A., Elena, S. F., Domingo, E. & Holland, J. J. (1994) *J. Virol.* **67,** 4295–4301.
29. Clarke, D. K., Duarte, E. A., Elena, S. F., Moya, A., Domingo, E. & Holland, J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4821–4824.
30. Duarte, E. A., Novella, I., Weaver, S. C., Domingo, E., Wain-Hobson, S., Clarke, D. K., Moya, A., Elena, S. F., de la Torre, J. C. & Holland, J. (1994) *Infect. Agents Dis.* **3,** 201–214.
31. Nagylaki, T. (1992) *Introduction to Theoretical Population Biology* (Springer, Berlin), pp. 5–7.
32. de la Torre, J. C. & Holland, J. J. (1990) *J. Virol.* **64,** 6278–6281.
33. Bennet. A., Dao, K. & Lenski, R. (1990) *Nature (London)* **346,** 79–81.
34. Lenski, R., Rose, M., Simpson, S. & Tadler, S. (1991) *Am. Nat.* **138,** 1315–1341.
35. Lenski, R. & Travisano, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6808–6814.
36. Gabriel, W., Lynch, M. & Burguer, R. (1993) *Evolution* **47,** 1744–1757.
37. Lynch, M., Burguer, R., Butcher, D. & Gabriel, W. (1993) *J. Hered.* **84,** 339–344.
38. Drake, J. W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7160–7164.